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Insight Into Skin Tumorigenesis Highlighting the Function of Epigenetic Regulators in SCC Formation

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14. ABSTRACT
Skin squamous cell carcinoma (sSCC) is one of the most common cancers worldwide. Chromatin regulators have recently emerged as one of the key players in tumorigenesis. Ezh2 is an essential component of the Polycomb complex that represses genes by compacting chromatin. Human genomics studies have shown that Ezh2 is overexpressed in epithelial cancers. Though it has been well accepted that Ezh2 repressive mechanisms depend on catalyzing H3K27me3, the key repressive histone modification mark, our data demonstrates that Ezh2 functions independently of H3K27me3. We found that Ezh2 expression decreased during calcium induced keratinocyte differentiation whereas H3K27me3 did not change. In addition, binding of Ezh2 at differentiation genes dramatically decreased while H3K27me3 maintained a sustained level of enrichment. Importantly ablation of Jmjd3 and UTX in the epidermis results in no obvious skin defects. On the other hand we have found that Ezh2 interacts with Fra2, which is one of important transcription factors in the AP1 family. Our findings suggest that Ezh2 may function in multiple ways to form a regulatory network and imply a new unexpected H3K27me3-independent mechanism. Deep understanding of this fundamental question will shed light on SCC formation.

15. SUBJECT TERMS
Ezh2, H3K27me3, stem cell, Skin squamous cell carcinoma

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Introduction

Skin squamous cell carcinoma (sSCC) is one of the most common cancers worldwide [1-3]. While sSCC is not a major source of mortality due to the relative ease of surgical resection of the tumor, this therapeutic strategy is the cause of considerable morbidity and cosmetic disability. Moreover, in patients with metastatic sSCC, the prognosis is poor, with only a 10–20% survival rate over 10 years[4]. Thus, a better understanding of the early steps of sSCC formation is essential to uncover novel and non-invasive therapeutic targets.

Chromatin regulators have recently emerged as one of the key players in tumorigenesis[5]. Increasing evidence indicates that the key chromatin repressor Ezh2 is an important regulator of tumorigenesis. Ezh2 is an essential component of the Polycomb complex that represses genes by compacting chromatin. Polycomb proteins form chromatin remodeling complexes referred to as Polycomb Repressive Complexes (PRCs)[6]. Comprised of Ezh2, Eed, and Suz12, PRC2 is recruited to chromatin, where Ezh2 catalyzes H3K27 methylation [7]. This histone mark signals to recruit PRC1 [7, 8], which aids in Polycomb-mediated repression [6, 9, 10].

Human genomics studies have shown that Ezh2 is overexpressed in epithelial cancers including prostate, breast, lung, and skin[11], but the molecular mechanisms by which Ezh2 promotes tumorigenesis are unknown.

Recently some evidence has indicated that the H3K27me3 and Ezh2 are not correlated in tumorigenesis, though it has been well accepted that H3K27me3 is the key repressive histone modification mark that is mainly catalyzed by Ezh2. EZH2 expression was high in glioblastoma (GBMs). Sequencing of GBMs showed H3F3A K27M mutations with lowered/absent H3K27me3. However, no significant differences in EZH2 expression were observed between H3F3A K27M mutant and wild type GBMs, suggesting that EZH2 may play an important role during tumorigenesis independent of H3K27me3 [12]. How Ezh2 plays its role is a fundamental question in the cancer field.

Our previous work presented in the original proposal indicated that ablation of Ezh2 leads to loss of H-Ras induced SCC formation. However, these past experiments did not address whether these effects are due to Ezh2-mediated methylation of histone H3 or due to some novel functions of Ezh2. Thus deep understanding of this fundamental question will shed light on the SCC formation. During the first year of my DOD fellowship, I focused my studies on addressing the following objectives:

1) Determine the relationship between Ezh2 and H3K27me3 in regulation of gene control in skin cells

2) Uncover the Ezh2 regulatory network by performing ChIP and protein interactome approaches.

Body

Part 1. Ezh2 exhibits H3K27me3-independent function during epidermal differentiation

1. We started by analyzing the expression of Ezh2 and global level of H3K27me3 during calcium-induced differentiation. To observe dynamic changes the assay includes four time points at 0, 24, 48 and 72 hours after exposure to increased calcium. As expected, the differentiation marker K10 was well induced during keratinocyte differentiation. We found that Ezh2 expression was drastically decreased by 48h but that the global level of H3K27me3 was sustained during differentiation. These results demonstrate that expression of Ezh2 may not be intrinsically linked to the presence of H3K27me3 in epidermal cells, implying that Ezh2 may have an important H3K27me3-independent function (Fig1A). We were also able to perform cell cycle analysis during differentiation and observed that keratinocytes exit the cell cycle at 48h (Fig1B) coinciding with the dramatic reduction in Ezh2 levels, indicating that Ezh2 controls keratinocyte stem cell state.

To further investigate how Ezh2 affects keratinocyte differentiation we performed ChIP for Ezh2 and H3K27me3 at differentiation genes including Keratin 1 (K1), loricrin (lor), and Filaggrin (Flg). Similar to global H3K27me3 expression levels, the enrichment of H3K27me3 was kept at nearly the same density and sustained at a considerable level at 72h, even though the expression of these genes is highly induced. Conversely, Ezh2 enrichment on differentiation genes decreased during keratinocyte differentiation (Fig1C). This discrepancy strongly suggests that Ezh2 has some function that is partially independent of H3K27me3. To fully understand the relationship between Ezh2 and H3K27me3, we have sent these ChIP samples for high-throughput sequencing. The ChIP-sequencing data will be carefully analyzed and will help us pinpoint where H3K27me3 and Ezh2 act on the genome level.

2. Analysis of Jmjd3 and UTX null skin yielded no obvious epidermal phenotype

Our in vitro data have shown that Ezh2 may play a role independently of H3K27me3. As the mechanism for maintaining stem cell state would be novel for Ezh2, we want to elucidate how H3K27me3 affects the epidermis. Jmjd3 and Utx are the only two known H3K27-specific demethylases. We thus generated skin specific

Jmjd3 and UTX double knockout mice using the K14-Cre system. Strikingly the double KO mice have no obvious phenotypes in the epidermis. Using immunofluorescent staining we observed that neither the undifferentiated basal layer marker K5 nor the differentiated suprabasal layer markers K1 and Lor had obvious defects when compared to wild type littermate mice (Fig2). This is unexpected because ablation of Ezh2 leads to premature differentiation [13], and we would expect the inability to demethylate H3K27me3 to result in an inability to differentiate. Therefore, our observations *in vitro* and *in vivo* have demonstrated that Ezh2 may contribute to stem cell fate independently of H3K27me3. This is a completely unexpected observation and our further investigation will be focused on this discovery.

3. Forced expression of Ezh2 cannot prevent keratinocyte differentiation

We subcloned Ezh2 into the pMIG retroviral vector and, in addition, also generated two loss-of-function mutants, Ezh2ΔNLS and Ezh2H689A. We transduced viruses for pMIG-Ezh2 and the two mutants in keratinocytes and observed successful overexpression of each (Fig.3). When these cells were exerted to calcium-induced differentiation, analyses showed that all these cells underwent differentiation properly when compared to control cells (Fig.3). We speculate that this is because most of the important components interacting with Ezh2, such as Suz12 and other transcription factors are not simultaneously highly expressed. Therefore, the interactome of the Ezh2 regulatory network needs to be fully investigated.

Part 2. Interactome of Ezh2 regulatory network

Existing data related to the role of Ezh2 demonstrates that its binding on the genome is important to the control of stem cells. Our previous results have shown that Ezh2 controls the proliferative potential of basal progenitors by repressing the Ink4a-Ink4b locus and tempers the developmental rate of differentiation by preventing premature recruitment of the transcriptional activator AP1 to structural genes that are required for epidermal differentiation [13]. Recently some evidence has shown that protein-protein interactions of Ezh2 offer another way to regulate stem cell state. Here we have proven that Ezh2 interacts with Fra2, which is the Fos-related AP1 transcription factor (Fig4). To further understand the biological importance of this

interaction we have established collaboration with Erwin Wagner to investigate Fra2 loss- and gain-of-function mice.

So far little is known about the EZH2-interacting proteins active in undifferentiated and differentiated stem cells or in specific cell lineages. Thus it will be more meaningful to extend these studies to achieve a high throughput outcome. We are preparing to identify these EZH2-interacting proteins using proteomics approaches.

Difficulty

Our main difficulty in this study to date was the Ezh2 antibody. We had tested and obtained one very good Ezh2 antibody from Active Motif. Unfortunately, soon after our study began this antibody was discontinued. We were forced to test a series of Ezh2 antibodies from multiple suppliers, including Active Motif, Millipore, Abcam etc., but we were unable to obtain comparable data to our original, even though most of them have been verified for ChIP-Seq. Finally, we have obtained an Ezh2 antibody (#5246) from Cell Signaling and have tested it against our original antibody with similar results and efficiency. Thus, we are quite sure that the data we have obtained are trustworthy, and we foresee no further problems going forward.

Key Research Accomplishments

- First we carefully characterized the Ezh2 and H3K27me3 expression pattern and, in addition, deeply investigated their dynamic enrichment on differentiation genes with ChIP during keratinocyte differentiation.

- Moreover, we have shown that epidermal-specific ablation of Jmjd3 and UTX, the only two known H3K27-specific demethylases, has no obvious defects.

  In vitro and *in vivo* our results demonstrated that Ezh2 might play roles independently of H3K37me3. Also the ChIP-Seq of Ezh2 and H3K27me3 is ongoing.

- We also started dissecting the Ezh2 regulatory network at the protein level. We have already confirmed the interaction of Ezh2 and Fra2, and the protein interactome of Ezh2 is in the pipeline.

Reportable Outcomes

Bardot ES, Valdes VJ, Zhang J, Perdigoto CN, Nicolis S, Hearn SA, Silva JM, Ezhkova E: Polycomb subunits Ezh1 and Ezh2 regulate the Merkel cell differentiation program in skin stem cells. EMBO J;32:1990-2000.

Conclusion

1. Ezh2 expression decreased during calcium induced keratinocyte differentiation whereas H3K27me3 did not change. These *in vitro* data suggested that Ezh2 might play roles partially independently of H3K27me3.

2. During keratinocyte differentiation enrichment of Ezh2 at differentiation genes dramatically decreased while H3K27me3 maintained a sustained level of binding.

3. We have proven that Jmjd3 and UTX are dispensable in epidermal development. Ablation of Jmjd3 and UTX in the epidermis results in no obvious skin defects.

4. Overexpression of Ezh2 cannot prevent keratinocyte differentiation, which suggests that Ezh2 functions in a regulatory network.

5. Finally I tried to decipher the protein interactome of Ezh2 regulatory network. We have found that Ezh2 interacts with Fra2, which is one of important transcription factors in the AP1 family.

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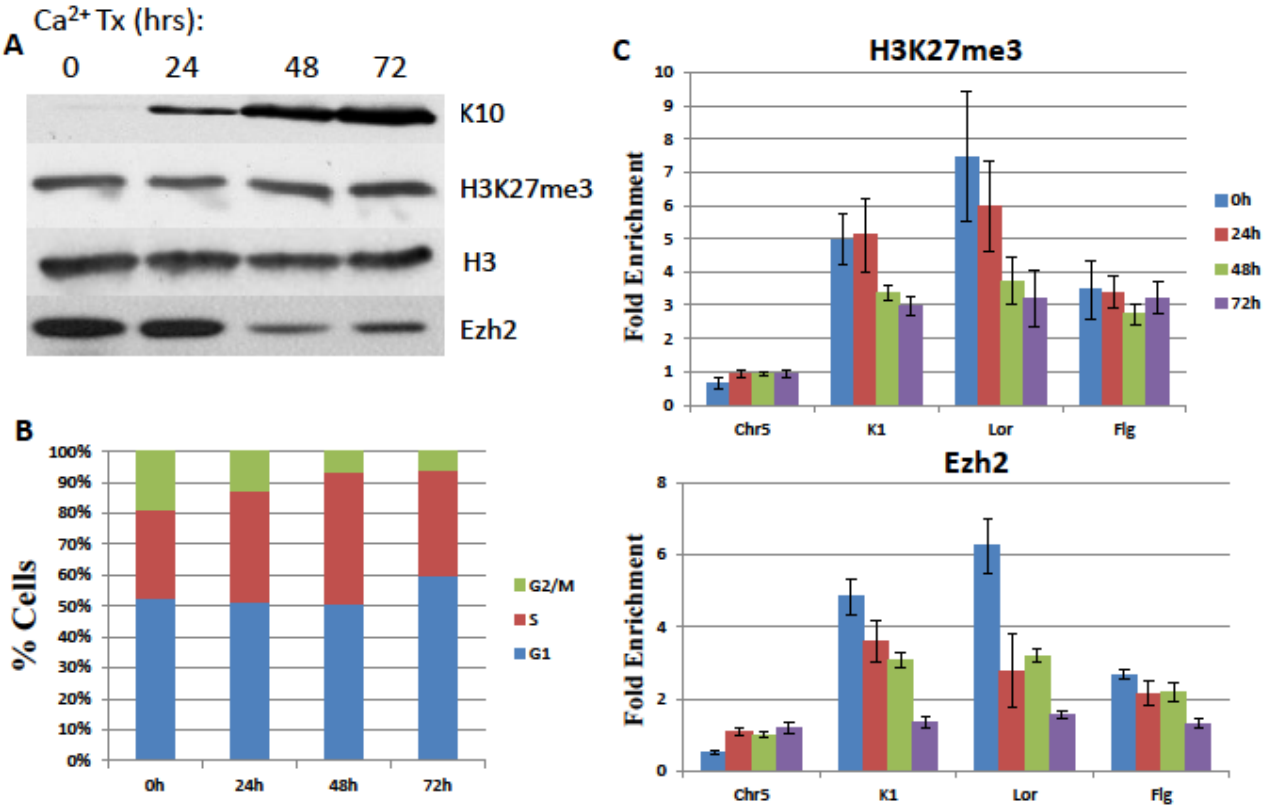
Supporting Data

Figure1



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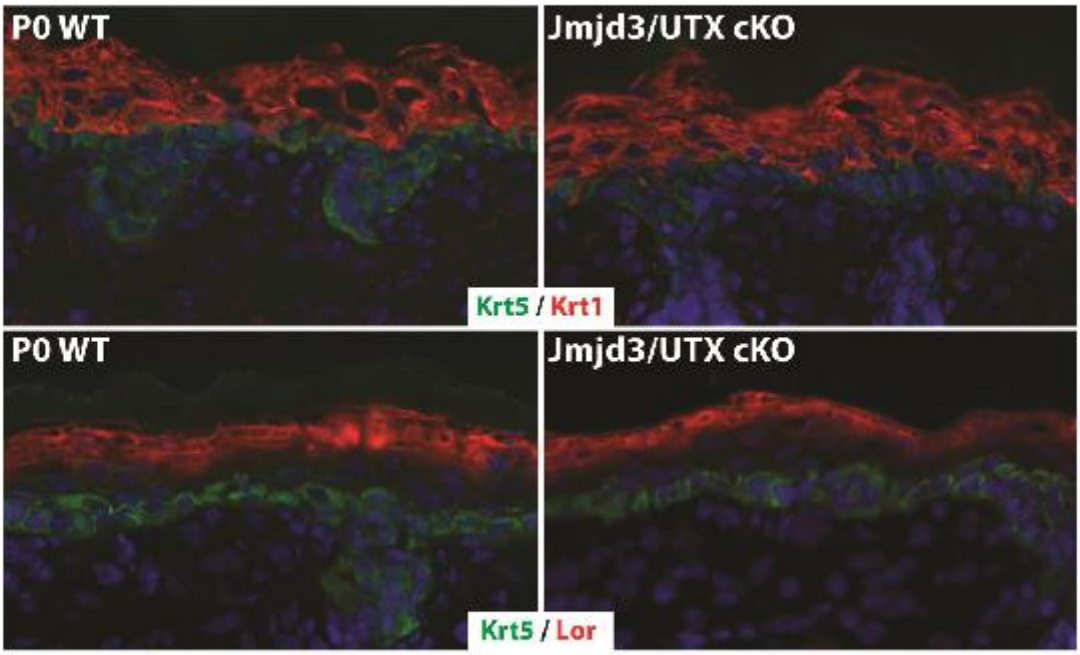

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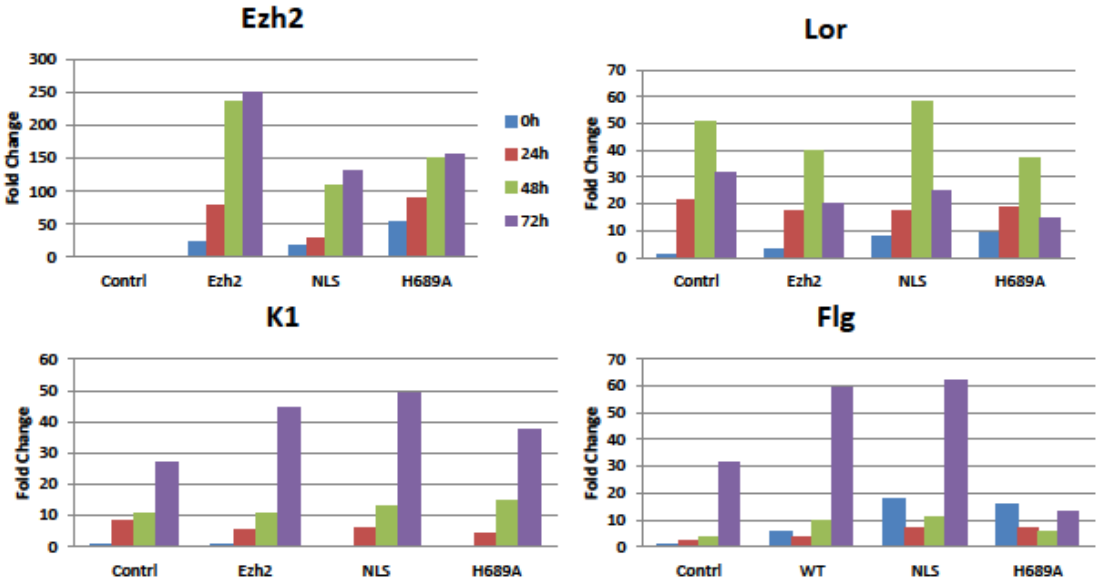
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Figure4

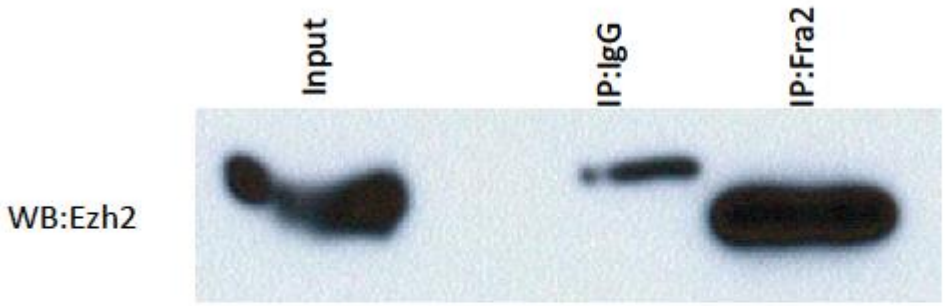

Figure Legends

Figure1. Ezh2 exhibits H3K27me3-independent function during epidermal differentiation. (A) Western blot analysis at indicated times confirming the expression of Keratin10 (K10), H3K27me3 and Ezh2 during calcium induced keratinocyte differentiation. Histone H3 (H3) was used as a loading control. (B) Cell-cycle distributions were determined using standard ethanol fixation and DAPI staining followed by flow cytometry. The keratinocyte differentiation time is as indicated. (C) ChIP assays were performed with H3K27me3 (upper panel) and Ezh2 (lower panel) antibodies. Quantitative levels of ChIP assays were analyzed by real-time PCR after normalizing with the input DNA and H3. PCR was performed with primers specific for the transcription start site of differentiation genes. Chr5 was used as negative control. Error bars represent standard error of the mean (SEM).

Figure2. Jmjd3 and UTX null skin yielded no obvious epidermal phenotype. Immunofluorescent (IF) staining of P0 WT and Jmjd3/UTX 2KO skin with antibodies against epidermis undifferentiated layer marker Keratin5 (K5) and differentiated layer markers Keratin1 (K1) and Loricrin (Lor) shows no obvious epidermal defects.

Figure3. Forced expression of Ezh2 cannot prevent keratinocyte differentiation. Ezh2 and two loss-of-function mutants, ΔNLS and H689A, were overexpressed in keratinocytes (upper left panel). The expression of keratinocyte differentiation markers Ketatin1 (K1), Keratin10 (K10), and Loricrin (Lor) was not impaired. The differentiation time course is as indicated.

Figure4. Co-immunoprecipitation (IP) studies of Ezh2 and Fra2 in keratinocytes. Whole-cell extracts were used for immunoprecipitation with an anti-Fra2 antibody, then immunoprobed with an anti-Ezh2 antibody. IgG was used as negative control. Input: unprecipitated extracts.